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Comparative study of the developed chemiluminescent, ELISA and SPR immunoassay formats for the highly sensitive detection of human albumin

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Abstract

Chemiluminescent enzyme immunoassay (CLEIA), surface plasmon resonance (SPR) immunoassay and enzyme-linked immunosorbent assay (ELISA) were developed for the highly sensitive detection of human albumin (HA). The bioanalytical procedure, involving the surface modification and antibody immobilization, was the same for all immunoassay formats. The bioanalytical platforms, i.e. microtiter plates (MTP) and SPR gold chips, were initially functionalized with 3-aminopropyltriethoxysilane and then crosslinked to anti-HA antibodies using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride and sulfo-*N*-hydroxysuccinimide. The developed HA immunoassay formats were compared on the basis of their analytical performance. CLEIA was found to be the best format for HA detection as it had the highest analytical sensitivity with lowest limit of detection and widest dynamic range. The analytical sensitivity of various immunoassay formats were in the decreasing order of CLEIA > ELISA > SPR. The developed CLEIA for HA detection was 6-fold more sensitive than the widely used commercially-available ELISA. The anti-HA antibody bound MTPs, stored at 4°C in 0.1M PBS, pH 7.4, were stable for up to 4 weeks, and can be effectively used for the rapid detection of HA in just 2.5 h.

Keywords: human albumin; CLEIA; SPR; ELISA;conventional; APTES; EDC; SNHS.

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1. Introduction

The precise determination of human albumin (HA) concentration is important for biomedical diagnostics and for monitoring the efficacy of treatment regimens. Human serum albumin (HSA) is a soluble monomeric protein produced in the liver. It is the most abundant protein in human blood plasma, which comprises about half of the blood serum protein. The HSA concentration in normal humans is between 25-50 mg/mL. The HSA levels are decreased in case of kidney disease [1], liver disease [2], inflammation [3], malnutrition [4], shock [5], nephrotic syndrome, diabetic nephropathy, burns, malabsorption, malignancy, genetic variations, rheumatoid arthritis [6], late pregnancy, and cardiovascular morbidity and mortality [7]. However, the increased HSA levels are almost always due to dehydration [8]. HSA is important in regulating the blood volume by monitoring the colloid osmotic pressure and buffering pH of the blood. It also transports many small hydrophobic molecules [9] such as bilirubin, progesterone, fatty acids, uric acids, calcium, antibiotics and various drugs in the blood.

The increased urinary albumin levels in diabetics indicate increased risk of developing end-stage renal and cardiovascular diseases [10]. It is also an indicator of the renal damage due to nephrotoxic substances [11]. Micoalbuminuria is a predictive marker for diabetic nephropathy. Therefore, the urinary albumin levels of diabetics are periodically monitored about three times a year so that the treatment can be immediately started after the detection of early stage renal damage. The detection of trace contamination of HA in pharmaceutical and other biological products is also equally important for monitoring the quality of in-process streams and final product, and for the development of optimized purification process.

Several analytical techniques, based on the use of piezoelectric quartz crystal [12], capillary electrophoresis [13], fluorescent dyes [14], ELISA [15], microfluidics [16], total internal reflected resonance light scattering [17], radial immunodiffusion [18], immunonephelometry [19], radio immunoassay [20] and immunoturbidimetric method [21], have been employed for the determination of HA. ELISA, SPR and CLEIA are the most commonly used immunoassay formats for disease diagnosis. ELISA has been the most widely used format for disease diagnosis from the last four decades, which is still being used on a very large scale due to its convenience, high sensitivity and specificity. On the other hand, the use of CLEIA and SPR immunoassay for disease diagnosis has increased considerably during the last decade. CLEIA provides very high assay sensitivity based on the availability of several highly sensitive chemiluminescent substrates [22], whereas SPR based instruments enables the rapid, real-time, and label-free analyte detection in addition to providing the highly useful information about biomolecular interactions. CLEIA and ELISA are quite similar as they involve the enzyme-mediated signal development. In ELISA, the enzyme-substrate reaction between HRP and TMB leads to the development of colorimetric signal. On the other hand, in CLEIA, luminol is used as a chemiluminescent material, which is activated by the enzyme-substrate reaction between HRP and H_2O_2, and oxidized to 3-aminophthalate to produce the desired chemiluminescence in its transition states.

The screening of an appropriate immunoassay format for analyte detection is very important for a particular bioanalytical application. Therefore, the main objective of this study is to compare the developed CLEIA, ELISA and SPR immunoassay formats for the detection of HA. The same assay components present in the commercially-available HA ELISA kit from Bethyl Labs, USA were employed for all the formats. The surface modification and antibody-crosslinking procedures were also identical for all the formats, where anti-HA antibodies were crosslinked using 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and sulfo-*N*-hydroxysuccinimide (SNHS) to the 3-aminopropyltriethoxysilane (APTES)-functionalized bioanalytical platforms. The developed CLEIA was found to be the best immunoassay format for HA detection and the most sensitive immunoassay for HA detection.

2. Material and Methods

2.1 Materials

Phosphate buffered saline (PBS, 0.1M, pH 7.4), 2-(N-morpholino)ethanesulfonic acid (MES, pH 4.7), EDC, SNHS, bovine-serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB) substrate kit, Supersignal ELISA femto maximum sensitivity substrate and LumiNunc^{TM} 96-well MTP for chemiluminescence assays were bought from Thermo Scientific. APTES, absolute ethanol, potassium hydroxide (KOH), Tween 20, Nunc 96-well flat bottom MTPs, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), H_2O_2 [30%, (v/v)] and H_2SO_4 [97.5% (v/v)] were procured from Sigma-Aldrich. The HA kit containing all sandwich ELISA components was procured from Bethyl Labs, USA. Anti-HA antibodies and horseradish peroxidase (HRP)-labeled anti-HA antibodies were employed as capture and detection antibodies, respectively. Ultrapure water (UPW) (18 MΩ, Direct Q, Millipore) was used for preparing buffers, KOH and 3-APTES; 0.1M MES buffer, pH 4.7 was employed to reconstitute EDC and SNHS; ELISA coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) was used for diluting anti-HA antibodies; and, sample/conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.005% Tween 20) was used for reconstituting HA and HRP-labeled anti-HA antibodies. ELISA coating buffer (0.05 M carbonate-bicarbonate, pH 9.6), ELISA wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), ELISA blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) and sample/conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.005% Tween 20) were purchased from Bethyl Labs, USA. The thermostat was procured from Labnet International Inc., USA, while Tecan Infinite M200 Pro MTP reader was bought from Tecan GmbH, Austria.

SPR was performed on BIAcore 3000 instrument from GE Healthcare, Uppsala, Sweden. The surface interaction analysis (SIA) kit Au (BR-1004-05) containing SPR Au chips were purchased from GE Healthcare. The SPR Au chip was assembled according to the instructions supplied by the manufacturer. 10mM HEPES buffered saline (HBS) buffer, pH 7.4 was employed as the running buffer for BIAcore. It was prepared, filtered through 0.2µm Millipore filter paper and degassed overnight in order to remove any air bubbles, which may cause interference with SPR analysis. All the sample dilutions were made in the running buffer (10mM HBS, pH 7.4). The dilutions of HA were made in BSA-preblocked glass vials, prepared by incubating with 1% (w/v) BSA for 30 min, to minimize analyte loss due to non-specific adsorption on sample tube surfaces and/or altered immunogenicity [23].

2.2 Surface modification of microtiter plate and crosslinking of antibody

Each well of the 96-well plate was treated with 100 µL of absolute ethanol for 5 min at 37°C and washed five times with 300 µL of UPW. Subsequently, each well was treated with 100 µL of 1.0% (w/v) KOH at 37°C for 10 min followed by five washings with 300 µL of UPW. The KOH-treated wells were then functionalized with amino groups by incubating with 100 µL of 2% (v/v) APTES per well at room temperature (RT) for 1 h inside a fume hood. The amine-functionalized plate was washed five times with 300 µL of UPW to remove excess of unbound 3-APTES from the surface. Thereafter, anti-HA antibody (990 µL of 10 µg/mL) was incubated with a 10 µL of premixed solution of EDC (4 mg/mL) and SNHS (11mg/mL) for 15 min at 37°C. 100 µL of the resulting EDC-SNHS activated anti-HA antibody solution was added to each of the APTES-functionalized wells followed by incubation for 1 h at 37°C. The anti-HA antibody-coated wells were then washed five times with 300 µL of ELISA wash solution.

2.3 ELISA

The anti-HA antibody-bound MTP were blocked with ELISA blocking solution for 30 min at 37°C and subsequently washed five times with 300 µL of ELISA wash solution. 100 µL of each of the varying concentrations of HA (6.25-400 ng/mL) were incubated in the anti-HA antibody-coated plates for 1 h at 37°C followed by washing with 300 µL of ELISA wash solution five times. 100 µL of HRP-labeled anti-HA detection antibody (diluted 1:75,000 i.e. 13.3 ng/mL) was then added and incubated for 1 h at 37°C followed by five washes with 300 µL of ELISA wash solution. The TMB substrate was then added (as per the manufacturer's guidelines) and the enzyme-substrate colorimetric reaction was allowed to develop in the dark for 20 min. The enzyme-substrate reaction was stopped by adding 100 µL of 0.18 M H_2SO_4. The absorbance was measured at 450 nm with reference at 540 nm. All the experiments were done in triplicate with zero ng/mL HA (in sample/conjugate diluent) as control, whose absorbance was subtracted from all the assay values. The conventional sandwich ELISA procedure was followed as per the manufacturer's guidelines.

2.4 CLEIA

The CLEIA procedure was similar to that of ELISA till the binding of HRP-labeled anti-HA detection antibody. However, CLEIA employs chemiluminescent substrate instead of TMB substrate that is used in ELISA. The working solution, made by mixing equal parts of luminol/enhancer and stabilizer peroxide solution, was subsequently added and incubated for 5 min. The chemiluminescent intensity was measured with Tecan Infinite M200 Pro's chemiluminescence readout at 425 nm. All the experiments were carried out in triplicate. The control for this study was zero ng/mL concentration of HA (in sample/conjugate diluent), whose reading was subtracted from all the assay values. The conventional CLEIA procedure was exactly similar to that of conventional ELISA with the exception of enzyme-substrate reaction.

2.5 SPR immunoassay

SPR based HA immunoassay was performed using our previously developed covalent procedure [24]. The cleaned SPR Au chip was incubated with 100 µL of 2% (v/v) APTES for 1 h at RT in a fume hood and washed extensively with DIW. EDC-activated anti-HA antibody was prepared by incubating anti-HA antibody [990 µL of 100 µg/mL in 10 mM HEPES buffered saline (HBS), pH 7.4] at RT for 15 min with 10 µL of cross-linking solution containing EDC (4 mg/mL) and sulfo-NHS (11 mg/mL) in 0.1 M MES, pH 4.7. Fifty microliters of EDC-activated anti-HA antibody was then injected over all the flow cells of APTES-functionalized Au chip at 10 µL/min. The non-specific binding sites on the chip were blocked by treatment with 20 µL of 1% (w/v) BSA. The change in SPR response units (RU) for the blanks were obtained by passing 50 µL of 10 mM HBS, pH 7.4 through all the flow cells. Thereafter, 50 µL of HA at seven different dilutions (6.2, 12.5, 25, 50, 100, 200 and 400 ng/mL) were passed through the flow cells. The change in RU for the blanks were subtracted from the change in RU for captured HA of corresponding flow cells. The most widely used carboxymethyl (CM5) dextran chip based immunoassay procedure, as described previously [24], was employed as conventional SPR immunoassay for HA.

2.6 Data analysis

SigmaPlot software, version 11.2 was employed to plot the immunoassay curves of developed CLEIA, ELISA and SPR immunoassays for HA detection using a four-parameter logistic function based standard curve analysis. The EC_{50}, Rsqr and hillslope were determined from the software report, while the

analytical sensitivity was calculated by [mean absorbance of blank + 3(standard deviation of the blank)]. The variability of the assay was reported as percentage coefficient of variation (%CV), while the intra-assay and inter-assay variability were determined from five assay repeats (in triplicate) on a single and five different days, respectively.

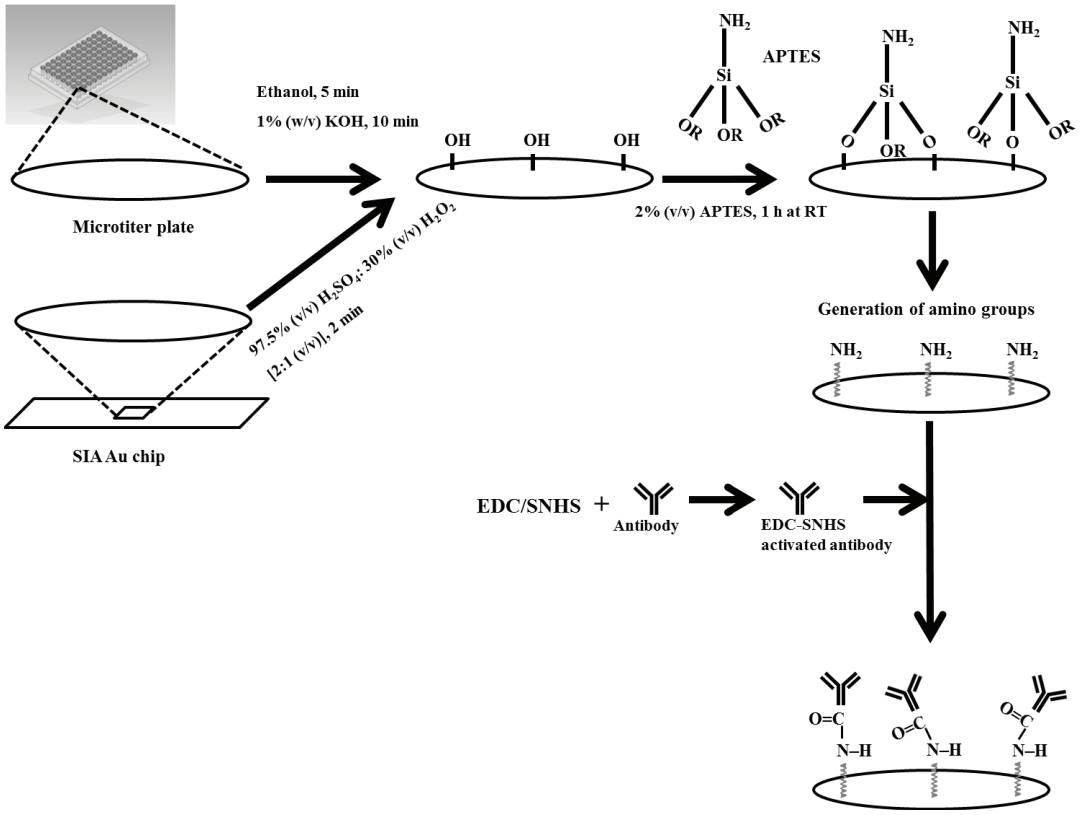

Fig. 1. Schematic of the bioanalytical procedure employed for the developed HA immunoassay formats for the covalent binding of capture anti-HA antibody to the APTES-functionalized bioanalytical platforms.

3. Results and Discussion

CLEIA, ELISA and SPR immunoassay formats for HA detection were developed using the same bioanalytical procedure that involved the silanization of substrates and covalent crosslinking of antibody. The various formats were then compared based on their analytical performance. Many studies have demonstrated that SPR immunoassays are comparable to ELISA in terms of analyte detection and sensitivity [25-27]. But they employed different surface modification and antibody immobilization procedures for ELISA and SPR, which added additional experimental parameters and thus, limited the actual analytical comparison of these techniques. Therefore, we have compared the various HA immunoassay formats by using the same bioanalytical procedure [Fig. 1] and assay components under the same assay conditions. The bioanalytical procedure employed for the surface modification and crosslinking of antibodies was derived by the customization of our previously developed procedure,

which had been used extensively by us for the development of rapid and highly-sensitive ELISAs [28-30], and SPR immunoassays [24, 31, 32].

As shown in Fig. 2 and Table 1, the developed CLEIA was the best immunoassay format for HA detection. It had wide dynamic range of 0.2-400 ng/mL with the lower limit of detection (LOD) of 0.3 ng/mL and the high analytical sensitivity of 1 ng/mL. The analytical sensitivity of various immunoassay formats were in the decreasing order of CLEIA > ELISA > SPR. The maximal half-effective concentration (EC50), which also corresponds directly to the sensitivity of an assay, followed the same trend i.e. CLEIA > ELISA > SPR. All the developed immunoassay formats were better than the respective conventional formats in terms of enhanced signal, wide dynamic range and high analytical sensitivity. This was mainly due to the leach-proof covalent crosslinking of anti-HA antibody to APTES-functionalized bioanalytical platforms that led to greater antibody immobilization density. The developed CLEIA for HA detection was 6-fold more sensitive than the widely used commercially-available conventional ELISA-based diagnostic kit. The inter-day and intra-day variability of the developed CLEIA for various concentrations of HA were between 2-15% and 1-11%, respectively, which were well within the acceptable range of less than 20% according to the bioanalytical method validation guidelines issued by US Food and Drug Administration (FDA).

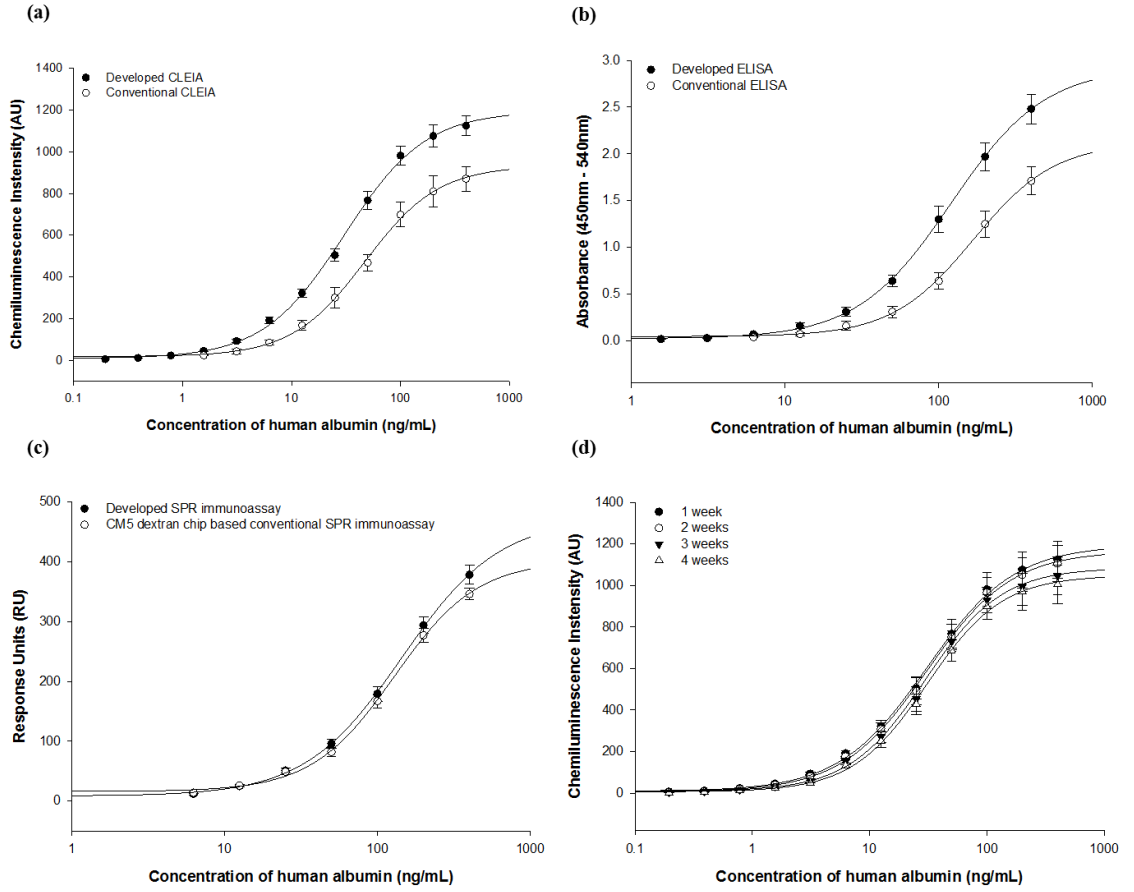

Fig. 2. Developed (a) CLEIA, (b) ELISA, and (c) SPR immunoassay formats, for the detection of human albumin. The CLEIA and ELISA formats were compared with the conventional passive-adsorbed antibody based formats, while SPR immunoassay was compared with the most widely used carboxymethyl (CM5)-dextran chip based conventional format. (d) Stability of the anti-HFA

antibody-bound microtiter plates for the detection of HA using the developed CLEIA format. All experiments were done in triplicate. The error bars represents standard deviations.

Table 1. Analytical comparison of the developed immunoassay formats for the detection of human albumin.

Analytical Parameters	**CLEIA**	**SPR**	**ELISA**	**Conventional ELISA**
Detection Range (ng/mL)	0.2-400	6.2-400	1.6-400	6.2-400
LOD (ng/mL)	0.3	7	1.6	3.2
Analytical Sensitivity (ng/mL)	1.0	12	3.0	6.2
EC_{50} (ng/mL)	31	145	121	215
% CV				
Intra-day	1-11	4-12	1-11	2-17
Inter-day	2-15	3-11	2-13	3-18

Table 2. A comparative analysis of the developed immunoassay formats for the detection of HA with various commercially-available HA ELISA kits.

Manufacturer	**Antibody immobilization technique**	**LOD***	**Refer to**
CLEIA	Chemical crosslinking	0.3 ng/mL	Reported here
ELISA	Chemical crosslinking	1.6 ng/mL	Reported here
SPR immunoassay	Chemical crosslinking	7 ng/mL	Reported here
Bethyl Laboratories, Inc., USA	Passive adsorption	3.2 ng/mL	Reported here
Alpha Diagnostic International, USA	Passive adsorption	10 ng/mL	http://www.4adi.com GenWay
Biotech, USA	Passive adsorption	4.11 ng/mL	http://www.genwaybio.com
Cayman, USA		250 ng/mL	http://www.caymanchem.com
Cygnus Technologies, USA		2 ng/mL	http://www.cygnustechnologies.com
Uscn Life Science Inc., China		1.23 µg/mL	http://www.uscnk.com
AssayPro, USA		0.39 µg/mL	http://www.assaypro.com
Biorbyt, UK		3.12 ng/mL	http://www.biorbyt.com
Antibodies-online GmbH, USA		4 µg/mL	http://www.antibodies-online.com
Innovative Research, Inc., USA		6.25 ng/mL	http://www.irbiologicals.com
Immunology Consultants Laboratory, Inc., USA		6.25 ng/mL	http://www.immunesystems.co.uk
Bio-Quant Diagnostic Kits, USA		5 mg/mL	http://www.bqkits.com
Abnova Corporation, Taiwan		3.12 ng/mL	http://www.abnova.com
CusabioBiotech Co., Ltd., China		78 µg/mL	http://www.cusabio.cn

Our results for SPR immunoassay and ELISA are complementary to the previous reports [33, 34] stating the higher sensitivity of ELISA in comparison to that of SPR immunoassay. But they contradict the findings of other reports, where SPR immunoassay was observed to be more sensitive than ELISA [35]. However, it is difficult to make a precise comparison as researchers have employed different assay steps and chemistries for these formats. Therefore, our results, based on the use of same bioanalytical procedure for various immunoassay formats, provide a more precise comparison of various analytical techniques. Our results demonstrating the higher sensitivity of CLEIA in comparison to ELISA agree

with the previous reports [36-38], where the LOD and sensitivity of CLEIA were 2-8 fold better than that of ELISA, when passively adsorbed antibody was used in both the formats. However, they are contrary to other reports [39], where there was no difference in the sensitivity and specificity of CLEIA and ELISA.

The anti-HA antibody bound MTP can be effectively stored at 4°C in 0.1M PBS, pH 7.4 for up to 4 weeks without any significant decrease in the chemiluminescent signal corresponding to the detection of HA by the developed CLEIA [Fig. 2(d)]. Therefore, the developed CLEIA is ideal for potential end-user applications in healthcare and industrial settings as it enables HA detection in just 2.5 h using prebound anti-HA antibody-coated MTPs. It is the best immunoassay format in comparison to developed ELISA, developed SPR immunoassay and widely used commercially-available conventional ELISA. To our knowledge, it is the most sensitive assay for HA detection [Table 2].

3. Conclusions

CLEIA, ELISA and SPR immunoassay formats were developed for the highly sensitive detection of HA using the same bioanalytical procedure, which involved the APTES-functionalization of bioanalytical platforms and the covalent crosslinking of anti-HA antibody. The various formats were compared on the basis of their analytical performance using the same assay components. The developed HA CLEIA was the best immunoassay format in terms of highest analytical sensitivity and widest detection range. The anti-HA antibody bound microtiter plates stored at 4°C in 0.1M PBS, pH 7.4 were stable for up to 4 weeks and enabled HA detection in just 2.5 h. The developed CLEIA is the most sensitive assay for the detection of HA.

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